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BY HIGH RESOLUTION ELECTRON MICROSCOPY

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W. H. Wu
(Ph.D. Thesis)

July 1983
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THE STRUCTURAL ANALYSIS OF THE SURFACE LAYER PROTEIN OF *Aquaspirillum serpens* BY HIGH RESOLUTION ELECTRON MICROSCOPY

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I would want to dedicate this piece of work to my mother.
ABSTRACT

*Aquaspirillum serpens* possesses a surface layer protein which exhibits a regular hexagonal packing of the morphological subunits (HP-protein). A method was developed to isolate large quantities of outer wall fragments of *Aquaspirillum serpens*, which appeared to be dark patches with symmetrical optical diffraction patterns. The outer wall fragments were manipulated to form large, light arrays with good crystalline order, of which the optical diffraction patterns always show "handedness" (i.e., absence of mirror symmetry). I concluded that these light arrays were real single layer protein arrays, whereas the dark patches were closed outer wall fragments with 2 layers of protein, collapsed on the grid. Two models for crystal formation have been presented in this thesis.

Low dose, low temperature electron microscopy has been applied to the frozen hydrated specimens of HP-protein. Computer image processing was applied to selected areas of micrographs. A statistical analysis was done to determine the existence of peaks above a local background from the output of a "Lattice Look" program, up to 13.70 Å resolution, which was below the limit of the first zero of the contrast transfer function in all directions. For
those peaks identified, the adjusted phases, i.e., phases adjusted for the phase shift due to the search for one of the 6-fold symmetry axes as a phase origin, were determined and they were close to 0 or 180 as expected. A computer processed image of the frozen hydrated specimen was obtained with a resolution of 13.70 Å. It was also noted that 6.9 Å structural details were preserved and retrieved in the image of the frozen hydrated specimen.

A method was developed where the specimen was first negatively stained, air dried, and frozen in liquid nitrogen, and then transferred to the high resolution cold stage (the "NSFT" method). A computer processed image was obtained with a resolution of 13.70 Å for the specimen prepared by the "NSFT" method. It was noted that 5.7 Å structural details were preserved and retrieved in the image of the specimen prepared by the "NSFT" method. The reason why the "NSFT" method can give high resolution was discussed.

The computer processed images for the two cases, i.e., the frozen hydrated method and the NSFT method, appear to be fairly different. A mathematical model was derived to account for the differences. In the NSFT method it seems possible that both protein and stain contribute to the image contrast, and interpretation of images can be difficult. A major advantage of the NSFT method is the fact that high quality micrographs can be taken with ease.
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CHAPTER 1

INTRODUCTION

1.1. Bacterial surface layer proteins

One of the most remarkable features of a variety of bacteria is the presence of regular arrays of macromolecules on their outer surfaces. Regularly patterned surface layers appear to be present in a large number of Gram-positive, Gram-negative, Gram-variable and halophilic bacteria, as well as thermophiles and acidophiles [Glauert and Thornley, 1969; Sleytr, 1978; Baumeister and Kubler, 1978; Beveridge, 1979; and Taylor, Deatherage and Amos, 1982].

The appearance of typical Gram-positive cell envelopes, as seen in thin sections, is illustrated in Fig. 1-1 [Sleytr, 1978]. A typical Gram-positive cell wall appears as a 15- to 80-nm thick, fairly homogeneous, electron-dense layer. An additional external layer composed of regularly arranged subunits (the surface layer) has been observed in numerous organisms. Underneath the cell wall is the plasma membrane, which is approximately 8 nm wide and has the typical structure of a unit membrane, consisting of two dense layers separated by a less dense layer.

In contrast to Gram-positive cell walls, Gram-
Fig. 1-1. Schematic diagram of the cell envelope of Gram positive bacteria as seen in thin section. The symbol "pm" designates the plasma membrane, which is seen as a trilamellar structure; "w" designates the cell wall and "s" designates the surface layer which has been observed in numerous organisms.
negative cell walls appear multilayered. In thin section (see Fig. 1-2) [Sleytr, 1978] the envelope typically shows the following layers: the plasma membrane (pm), seen as a unit membrane, the dense layer (d), in which the peptido-glycan is located, the intermediate region, and the outer membrane (Om), which has a unit membrane structure similar to that of the plasma membrane. An additional external layer composed of regularly arranged subunits has been observed in numerous organisms.

The regular surface layer arrays of only a few bacteria have been isolated and chemically characterized. All of these have been shown to be proteinaceous in nature and, with the possible exception of the multilayered surface layers found in a few Gram-negative species, most are composed of a single polypeptide species.

The functions of the regular arrays of subunits on bacterial surfaces are still mainly a matter of speculation. Studies on the function of surface layers to date indicate that the function may be different in different species.

For many Gram-positive and Gram-variable organisms, it was speculated that the surface layer had a protective function [Buckmire, 1971; Thornley et al., 1974; Sleytr, 1976; Crowther and Sleytr, 1977]. Many Gram-positive and Gram-variable bacteria with surface layers have been shown to possess a relatively thin murein layer which has a
Fig. 1-2 Schematic diagram of the cell envelope of Gram negative bacteria as seen in thin section. The innermost layer is the plasma membrane (pm) which is covered by the cell wall. The cell wall consists of a dense layer (d), in which the peptidoglycan is located; an intermediate layer, an outer membrane (om), which has a unit membrane structure similar to that of the plasma membrane; and a surface layer (s) which has been observed in numerous organisms.
greater similarity to the murein layers of gram-negative than to those of typical gram-positive species [Glauert and Thornley, 1969]. It has been reported that in several cases removal or partial degradation of the surface layer can make the peptidoglycan layer susceptible to digestion by lysozyme [Nermut and Murray, 1967; Takumi and Kawata, 1970; Wallinder and Neujahr, 1971].

Extensive studies on gram-negative rumen bacteria have shown that all organisms appear to possess additional, partially regularly arranged, cell wall layers outside the outer membrane, and some of these seem to be composed of structural protein [Costerton et al., 1974; Cheng and Costerton, 1975]. It was suggested that the universality of this layer among rumen bacteria is related to their adaptation to life in a highly competitive environment. It was also suggested that these layers may function in adhesion of the bacterial cell to a surface or in conditioning the ionic and molecular environment for both cell wall and plasma membrane enzymes [Costerton et al., 1974].

Enzymatic activity of the surface layer subunits themselves was demonstrated by Thorne et al. [1976b], who found that the tetragonally arranged protein attached to the outer membrane of Acinetobacter sp. strain 199A has a phospholipase-A₂ activity. Half of the newly synthesized a-layer protein is secreted into the growth medium [Thorne
et al., 1976a], but the specific activity of the intact cell walls is less than one-tenth of that of the detached subunits. Thus the enzyme is only fully active in the free form and appears to function as an extracellular enzyme secreted for the hydrolysis of foreign phospholipids [Thorne et al., 1976b].

Based on morphological data, a complex secretory function was postulated for the hexagonally arranged goblet-shaped subunits attached to the outer membrane of the gliding bacteria F. polymorphus [Ridgway and Lewin, 1973]. These complex subunits were thought to be membrane-bound organelles or macromolecular pores through which extracellular polymeric material could pass to the outside of the cell. The secreted polymers may aid in attachment of the cell to the substratum or provide the propulsive force for gliding [Ridgway et al., 1975].

Surface-layer assembly systems may contribute in particular cases to the maintenance of cell shape. Such a cell envelope-stabilizing function of regularly arranged subunits was postulated for organisms that lack peptidoglycan, such as Halobacteria [Mescher et al., 1974; Mescher and Stromunger, 1976], S. acidocaldarius [Weiss, 1974], and the intracellular parasite Chlamydia psittaci [Matsumoto and Manire, 1970].

Direct evidence for a protective function of surface layers on a gram-negative organism was provided by
Buckmire [1971], who demonstrated that Aquaspirillum serpens\textsuperscript{1} strains that have regular surface layers are resistant to invasion by Bdellovibrio bacteriovorus. The removal of the surface layer made the cells susceptible to the endoparasite, and the reformation of the regular pattern was accompanied by a return of resistance. Martin et al. [1972] suggested that the regular surface layer may increase the rigidity of the outer membrane of Aquaspirillum serpens.

In the following section some of the previous work done on the surface layer protein of Aquaspirillum serpens will be reviewed. Motivations for studying this protein via high resolution electron microscopy will be discussed in section 1.3.

1.2. Previous work on the surface layer protein of Aquaspirillum serpens

A regularly patterned surface layer found on the wall of Aquaspirillum serpens was described by Murray [1963]. Aquaspirillum serpens was first isolated by Muller from fresh water in 1884. (Bergey's manual of Determinative Bacteriology, 1974) Cells are strictly aerobic. Cells observed by phase microscopy are 0.6-1.1 µm in diameter.

\textsuperscript{1} Aquaspirillum serpens was first termed Spirillum serpens. It later assumed the new name so as to be differentiated from Oceanospirillum serpens. (R. G. E. Murray, personal communication)
and cell length is in the range of 3.5-12 um (see Fig. 1-3). Cells are motile, possessing a tuft of flagella at the pole.

Murray [1963] found the hexagonally-packed surface array on the wall of *Aquaspirillum serpens* VHA. Negatively stained and shadowed preparations as well as sections of whole cells and of isolated cell wall fragments showed that this was the outermost of the cell wall layers and lay upon the triplet cell wall layer typical of Gram-negative bacteria [Murray, 1963]. The material to which the surface layer protein is attached has been referred to as a "backing layer" [Buckmire and Murray, 1970; 1973]. The en face view of the hexagonally packed (HP) protein layer shows globular subunits, which have an apparent hole in their center and which are attached to one another by a relatively faint, three-pronged connection (referred to in the literature as a "Y-linker" structure) [Buckmire and Murray, 1976]. The HP-protein can be released from the outer membrane by mild guanidine hydrochloride treatment [Buckmire and Murray, 1970]. The HP-protein attaches again to the outer membrane, in an ordered structure, on removal of the guanidine hydrochloride by dialysis and on addition of calcium ion [Buckmire and Murray, 1973, 1976]. However, the surface-layer protein of *Aquaspirillum serpens* does not form a two-dimensional array in the absence of a backing layer, under the same conditions [Buckmire
Fig. 1-3. Micrograph of a suspension of *Aquaspirillum serpens* taken with a light microscope using phase contrast optics.
and Murray, 1973, 1976].

A morphological model of the structure of the HP-protein has been proposed by Buckmire and Murray [1976]. This model assumes that the monomer molecular weight of the protein is about 48,000, and that these molecules are associated in trimers having a three-fold, spoke-like structure. The trimers in turn are associated with one another as hexamers to give a morphological unit consisting of a large globular structure with a central hole and six radial spokes, which constitute the Y-linkers seen in the intact structure. A special feature of this model is that three of the radial spokes or Y-linkers lie at one level in the structure and three lie at a second level.

Glaeser et al. [1979] have proposed an alternative morphological model in which the morphological unit was described as having the appearance of a flared-out, hollow cylinder with six "spokes" at the flared end (see Fig. 1-4). Use of this model makes it possible to interpret and account for a wide variety of side views that can be seen in negatively stained specimens as well as in frozen hydrated specimens [Glaeser et al., 1979]. The molecular weight of the morphological subunit can be estimated from the electron microscope measurements made on the unstained, hydrated material. The estimated molecular weight is consistent with the hypothesis that the morphological subunit is a hexamer of the ~140,000 dalton
Fig. 1-4. A morphological model of HP-protein proposed by Glaeser et al. [1979]. The morphological unit is shown to have the appearance of a flared-out, hollow cylinder with six "spokes" at the flared end.
peptide which is observed by gel electrophoresis [Glaeser et al., 1979].

1.3. Motivations for structural study of HP-protein by electron microscopy

From the thickness of HP-protein and the area it covers, it can be estimated that the HP-protein makes up at least 5% of the total protein the organism produces. Many interesting questions of general significance in cell biology are raised in connection with the morphogenesis of the regular arrays, especially the synthesis, transport, and assembly of the constituent subunits.

Detached surface layer subunits from many other gram-positive and gram-negative bacteria have been shown to possess the ability to assemble under appropriate conditions in the absence of any supporting layer. The two-dimensional crystals formed by these assembly processes have a structure identical to that of the regular arrays observed on intact cells. Self assembly experiments with all other surface layer proteins have shown that the patterns of the regular arrays seem to be determined only by the directional bonds between the subunits and not by any order in the underlying cell wall layer. This ability of the surface layer subunits to assemble freely into regular arrays and to adhere to suitable surfaces represents the most simple mechanism for keeping a growing
cell surface completely covered with a highly ordered monolayer of macromolecules.

However, Buckmire and Murray's work repeatedly led to the conclusion that in the case of HP-protein, self-assembly without a "backing layer" was not possible. This observation makes this protein unique and most interesting to study.

From my observation (see chapter 2), however, I have partial evidence to conclude, based upon pattern recognition of the electron images, that the HP-protein may indeed possess the ability to assemble spontaneously under the right conditions, to form regular arrays without supporting layers.

A high resolution, 3-dimensional structural analysis will give substantial insight about the detailed association between HP-protein subunits, as well as clarify the possible modes of interaction, if existing, of the surface protein with the underlying lipopolysaccharide and/or other components of the outer bacterial membrane.

As thin 2-dimensional crystals, surface layer arrays are most suitable for structural analysis via electron microscopy. The interaction of electrons with condensed material is about 100,000 times stronger than that of X-rays. A practical specimen for X-ray diffraction would be at least of the dimension of (100 micrometers)$^3$ while for electron diffraction a crystal of the dimension 1 micron x
l micron x 100 Å is perfect. Another advantage is the high efficiency in data collection compared with that of x-rays. The wavelength of 100kev electron is 0.037 Å, implying a large Ewald sphere radius compared to a typical X-ray wavelength 1.5 Å. In fact, one can assume a flat Ewald sphere without introducing serious error at sufficiently high resolution. A very important advantage to point out is, in electron microscopy, one does not have the phase problem as in the case of X-ray diffraction.

The fact that the surface layer protein is a thin object that is made up of light atoms enables one to approximate it as a "weak phase object". The "contrast transfer function" theory of image formation of such an object with coherent illumination is well established [Hanszen, 1971; Hoppe, 1970; Hoppe et al., 1970; Lenz, 1965, 1971], and the theory has been extended to include effects of partial spatial and temporal coherence, as well as other instrumental instabilities [Hanszen and Trepte, 1971a, 1971b; Frank, 1973]. This theory predicts a simple relationship between the Fourier transform of the object and that of the image:

\[
F[I_{\text{image}}(x,y)] = \frac{2}{\hbar c B} F[V'(x,y)] \sin(\hat{s}) E(\hat{s}), \text{ for } \hat{s} \neq 0
\]

Here \( I_{\text{image}}(x,y) \) is the image intensity, \( F \) is the Fourier transform operator, \( \hbar \) is \( 1/2\pi \) times Planck's constant, \( \hat{s} \) is the spatial frequency variable,
\[ V'(x,y) = \int V(x,y,z) \, dz \]

is the projected potential of the object, \( \gamma(\tilde{s}) \) is the phase distortion due to spherical aberration, \( C_s \), and defocus, \( \Delta z \) given by

\[ \gamma(\tilde{s}) = 2\pi \left[ C_s \lambda^3 s^4 / 4 - \Delta z \lambda s^2 / 2 \right] \]

where \( \lambda \) is the wavelength of the electrons, and \( E(\tilde{s}) \) is the envelope function due to partial spatial and temporal coherence, as well as instrumental instabilities. The Fourier transform of the image is thus equivalent to the Fourier transform of the object structure multiplied by the "contrast transfer function", \( \sin \gamma(s) \), and an attenuation function \( E(s) \).

With all the merits of electron microscopy in dealing with "thin" objects, one should carefully look into the problems of electron radiation damage and dehydration of biological specimens in vacuum. The following two sections summarize the problems and solutions.

1.4. Effects of electron radiation on biological specimens

One important limitation to high resolution electron microscopy of biological specimens is that of electron radiation damage. Several reviews discuss the effects of radiation on biological specimens, as well as methods to assess damage [Glaeser, 1975; Isaacson, 1975; Stenn and
Bahr, 1970; Reimer, 1975]. In the electron microscope, a small number of the electrons passing through the specimen will be inelastically scattered. As energy is transferred from the electrons to the specimen, excited, ionized and radical species are formed. These chemically active species will tend to combine with other charged ions or with electrons to reach a more stable state. The molecular changes that follow can be generalized into the following categories: (A) Bond dissociation and loss of small side groups, resulting in mass loss and fragmentation of larger molecules into smaller ones. (B) Formation of new bonds: cross-linking between neighbouring molecules or with a contiguous portion of the same molecule. (C) Changing of bond type: on losing hydrogen, an organic molecule acquires double and triple bonds. (D) The secondary and tertiary structures of organic molecules dependent on weak bonds (e.g., hydrogen bonds, hydrophobic interactions, and van der Waals bonds), and these bonds can be disrupted by the absorption of a small amount of energy (0.1 - 1 ev) [Stenn and Bahr, 1970].

Methods used to assess the extent of specimen damage include loss of or changes in crystalline diffraction [Glaeser, 1971; Siegel, 1972], electron energy loss spectroscopy [Isaacson et al., 1974], infrared absorption spectroscopy [Baumeister et al., 1976], and mass loss determinations by autoradiography or loss of specimen
The susceptibility of biological specimens to radiation damage demands that imaging must always be carried out with exposures not exceeding a certain critical value \( N_{cr} \), characteristic of the terminal stage of specimen damage.

The critical dose has been defined empirically as the dose at which either complete fading of high resolution diffraction spots occur or the dose at which the diffraction spots of interest fall to \( 1/e \) of their original value if the loss of structure follows an exponential decay [Glaeser, 1975; Hayward and Glaeser, 1979]. The critical exposures for some biological specimens have been established [Glaeser, 1971; Reimer, 1975], and they generally are not larger than a few electrons/\( \mu \text{m}^2 \) [see e.g. Glaeser, 1975].

The maximum resolution that can be obtained from a micrograph taken with such a "critical" exposure, \( N_{cr} \), is limited by the statistical nature of the electron events on the recording medium. The relationship between the attainable resolution \( d \) for a specified contrast \( C \) and the number of incident electrons per unit area \( n \) is given by the Rose equation [1948]:

\[
Cd > 5 / (n)^{1/2}
\]

The contrast \( C \) is defined as the difference in
intensity between two image points, separated by the distance \( d \), divided by the average intensity of the two points. The value of 5 in the numerator was determined by visual perception experiments. Since \( C \) is typically \(< 10\%\) for biological specimens, at the dose of \( N_{cr} \), i.e. about 1 electron/\( \AA^2 \), \( d \) is \( > 50 \\AA \). We see that high resolution imaging is impossible.

Two methods of enhancing attainable resolution are presented below:

1.4.1. SNAP-shot method

The SNAP-shot method [Kuo and Glaeser, 1975] utilizes the redundant information in the noisy image of a periodic object to reconstruct a statistically defined image of a single unit cell. The periodicity of the object can be determined from its Fourier transform, and a Markham-type real space superposition procedure [Markham, Frey and Hells, 1963] can be applied to get the statistically defined image. Alternatively, a delta-function filtering performed on the reciprocal lattice will yield the same spatially averaged image. The mathematical justification for the latter approach has been elucidated by Aebi et al. [1973]. If \( R \) is the number of repeating unit cells in the recorded image, the Rose equation gives

\[
d_s > 5 / C(Rn)^{1/2}
\]

where \( d_s \) is the resolution attainable by the SNAP-shot method.
method. In other words, if we image a periodic object such as the surface layer protein with a low exposure, we can superimpose many unit cells, and thus build up a statistically well defined image of one unit cell. If the equivalent dose is 400 times the value of $1 \text{ electron/Å}^2$ because of the superposition of 400 unit cells, then the minimum resolution possible drops from 50 Å to 2.5 Å.

1.4.2. Low temperature electron microscopy

Another line of approach in overcoming the specimen damage problem is to find means of enhancing the critical dose. Experimental results have shown that the critical dose is increased almost an order of magnitude, as measured from the fading of the electron diffraction pattern, if the specimen is kept at liquid nitrogen temperature instead of room temperature [Glaeser and Taylor, 1978; Hayward and Glaeser, 1979].

Taylor and Glaeser [1976] have suggested that the increase of the critical dose of frozen-hydrated catalase over room-temperature hydrated catalase is due to the support provided by the ice matrix. They believe that for a protein molecule in a liquid matrix, molecular rearrangement could occur quite easily, whereas if the water matrix is solid, then diffusion of molecular fragments could be in effect reduced, and the protein conformation might be maintained up to a higher dose of irradiation. They have
also suggested that the larger effect in the case of hydrated catalase is quite probably a reflection of differences associated with the liquid vs the solid state. They cited the threefold increase of the critical dose of glucose-embedded catalase at low temperature over that at room temperature as evidence for the fluidity of the embedding glucose at room temperature.

Hayward and Glaeser have shown that the critical dose of glucose-embedded purple membranes increased almost an order of magnitude, if the specimen is kept at liquid nitrogen temperature instead of room temperature [1979]. They reasoned that since it is doubtful that much of the volume of the purple membrane is taken up by water, and very little of the membrane lattice extends above the surface of the membrane [Henderson, 1975], the only glucose and accompanying bound water is probably on the surface of the membrane. They suggested that it is possible that a decrease in fluidity of the glucose on the surface would prevent radiolytic fragments from diffusing away from the membrane, and would prevent free radicals from diffusing across the surface of the membrane and causing further damage. They also suggested that it is possible that the membrane itself is less fluid at low temperature. Evidence for fluidity of single protein molecules at room temperature includes diffusion of oxygen through proteins [e.g., Lakowicz and Weber, 1973] and the availability of
"buried" side groups to fluorescence quenchers [Eftink and Ghiron, 1976]. They suggested that it seems possible that the diffusion of free radicals through the purple membrane lattice, and the displacement of radiolytic fragments within the membrane, could be substantially reduced at low temperature.

Having discussed approaches in overcoming the radiation damage problem, the author would like then to outline specimen preservation methods in the high vacuum of the electron microscope.

1.5. Preservation of biological specimen for electron microscopy

Throughout the history of electron microscopy there have been attempts to develop methods for maintaining the hydration necessary for biological specimens. The primary drawback of techniques for preserving specimen structure without maintaining specimen hydration is the fact that they do not preserve periodic structures in a full high resolution state or, even more seriously, they may alter the structure. Langer et al. [1974] and Hoppe et al. [1968] have shown by x-ray diffraction techniques, that the periodicity of certain protein crystals was reduced, in the best instance, to about 8 Å after they had been aldehyde fixed, dehydrated and embedded in various polymerizing agents. This is a significant reduction in dif-
fraction resolution for it is known that fully hydrated protein crystals can diffract x-rays to resolutions often greater than 2 Å. In addition, studies by Moretz et al. [1969a,b] have demonstrated that following fixation, dehydration and embedment of myelin, not only is the lattice dimension reduced but also the relative intensities are altered. These changes are strong evidence of the production of an artifactual structure. Many of the attempts, designed to replace the aqueous phase with something that has a lower vapor pressure at room temperature, may subject the specimens to a range of physical stresses which can explain the lack of preservation of the minute details of structure. The main exception to this statement, as mentioned in the previous section, is the glucose embedment technique of Unwin and Henderson [1975] which has led to the first views (projected and 3-dimensional) of a membrane protein at a resolution of approximately 7 Å. However, the possibility that glucose disorders some parts of the structure is still open [Jaffe and Glaeser, 1982], and evidence has been presented that high concentrations of sugar alter the membrane structure [Hsiao et al., 1978].

In this chapter I will outline some of the past attempts to determine the structure of various biological systems via different preparatory techniques.
1.5.1. Freeze drying and critical point drying techniques

In the freeze drying technique the first step is to flash freeze the material of interest. The rapidity of freezing is an important parameter. Slow freezing can lead to the formation of ice crystals which will submit the material to severe forces which may ultimately lead to deformation [Merryman, 1974]. In the next step, either the pressure can be lowered or the temperature of the specimen can be raised leading to the sublimation of the water. Using a method of spraying micro droplets onto a cooled surface to insure rapid freezing, Williams was able to show a substantial improvement over air drying in the preservation of red blood cell ghosts and intact bacteria.

Attempts have also been made to preserve catalase crystals via freeze drying for high resolution electron microscopy [Lepault and Dubochet, 1980]. Resolution obtained by these researchers has been limited to 8.5 Å in contrast to the resolution of 2.8 Å obtained using frozen hydrated crystals.

In the critical point drying technique the water in the biological material is first replaced by a liquid whose critical temperature and pressure are of a suitable nature. This is necessary because the critical temperature of water is 347°C which is too high to be of a practical use in the method. One implementation of the method
employs a series of ethyl alcohols of increasing concentrations. The alcohol is then replaced by amylacetate which, in turn, is replaced by liquid carbon dioxide. The critical temperature and pressure of carbon dioxide are 36.5°C and 1080 lbs/in², respectively. The specimen, immersed in liquid carbon dioxide under pressure, is then passed through the critical temperature of carbon dioxide. At or above this temperature the liquid and the gas become a single fluid phase, which can then be bled off. After release of the gas, the specimen, now dry, can be inserted into and viewed in the electron microscope.

This technique, like the freeze drying one, has been used very successfully on a moderate resolution basis. It was, however, observed that specimens prepared in this manner showed a characteristic type of dehydration artifact: flagella that were stretched between surfaces and allowed to be free between these surfaces were flattened at the point of surface contact [Anderson, 1954]. It was hypothesized that structures which are freely suspended under vacuum vibrate as a result of their thermal energy and collapse irreversibly upon the support.

An additional limitation of this technique in the case of membrane bound structures arises from the fact that the solvents that are used to replace water are strong protein denaturants. They may also extract lipids from the membranes.
1.5.2. Various hydration techniques

Due to the limitations of the preservation techniques described above, many researchers have tried to maintain the aqueous environment itself in the electron microscope. This can be accomplished by either lowering the temperature of the specimen and thus lowering the vapor pressure of the water, or by raising the column pressure in an environment local to the specimen.

In an effort aimed at the latter approach, a group led by Parsons [1974] was able to obtain electron diffraction patterns of catalase [Matricardi et al., 1972] to a resolution reported to be $2 \AA$. Specimens were kept hydrated by maintaining them in an atmosphere of saturated water. This was facilitated by building a small chamber in the microscope with 100 micrometer apertures on either side of it. Another layer of 200-400 micrometer apertures were placed around the first. The gas escaping from the inner set of apertures was pumped out of the pole piece gap by a liquid nitrogen trap and mechanical pump. The remaining gas was pumped away by the vacuum system of the microscope. Thus, this small set of apertures allows electron beam penetration but prevents large amounts of water from evaporating into the microscope column. There are several present limitations of the technique. One concerns the problem of Brownian motion at room temperature in noncrystalline specimens. Another one is that
there is presently no way to obtain high resolution phases via imaging, as no high resolution stage exists.

The glucose embedment technique of Unwin and Henderson [1975] allowed these authors to utilize their existing microscope facility to obtain room temperature diffraction patterns and images of purple membranes (PM) to 7 Å. The technique itself is quite simple and consists of mixing the sample with a 1 or 2% solution of glucose and allowing the mixture to dry down onto a carbon coated grid. The result of this procedure is that a reasonable number of PM's are covered with a layer of glucose of sufficient thickness to keep the crystalline membrane hydrated, but thin enough to be reasonably electron transparent. However, evidence has been presented [Hsiao et al. 1978] that high concentrations of sugar alter the membrane structure, that glucose embedment renders image interpretation difficult [Wah Chiu, private communication]; and that glucose might disorder some parts of the structure [Jaffe, Ph D thesis].

Taylor and Glaeser [1976] first showed that there was a large degree of image contrast as a result of embedding catalase in ice. Using a 100B JEOL microscope [Taylor and Glaeser, 1975] these authors were able to freeze specimens outside of the microscope and insert them at LN₂ temperature into the column. Excellent high resolution diffraction patterns of catalase were obtained as well as high
contrast images [Taylor and Glaeser, 1976]. This contrast is likely due to the density difference in the interior regions of catalase crystals that are filled with water versus the density of protein.

The resolution of the maps obtained using these techniques were primarily limited by the availability of high resolution phases. Mechanical instability of the specimens was observed which limited the resolution to around 12 Å [Taylor and Glaeser, 1976]. These problems were later overcome by moving the microscope to an environment with less vibration and by the construction of a high resolution cold stage [Hayward and Glaeser, 1980].

The method used by Taylor and Glaeser [1973] to prepare crystalline specimens consisted of preparing carbon films coated with a layer of silicon oxide. As this layer was hydrophilic it retained a layer of water. In an effort to control the rate of evaporation of water, folding grids were used to sandwich the specimen between two layers of carbon film. Excess water was drawn off with filter paper and specimens were immediately plunged into LN₂. Although some good quality diffraction patterns were obtained by using this method, reproducability was definitely a problem [Taylor, private communication].

In a subsequent modification of this technique, specimens in water were placed onto carbon coated grids and allowed to dry at a controlled rate in a humidity box.
[Taylor and Glaeser, 1976]. When grids were judged to be of an appropriate thickness as examined in a phase contrast optical microscope they were quickly frozen by insertion into LN$_2$. This method was more reliable than the previous one, however, reproducability was again a problem.

A monolayer technique was developed in order to utilize the known orientation of PM on polylysine as a function of pH. The specimen preparation developed by Hayward et al. [1978] was to take polylysine coated grids and apply PMs at a range of different pH. These grids were then placed into a solution of 5 mM CaCl$_2$ at pH 8-9. Several drops of castor oil were then applied to the surface followed by a drop of 2 percent stearic acid in hexane. As the hexane evaporated, a surface film of stearic acid was formed through which the submerged grid was withdrawn. Grids were then frozen in LN$_2$ and placed into a JEOL 100B microscope for diffraction which extended to approximately 4 Å. Catalase was also prepared via this method as a test specimen and some partial disordering was evidenced on diffraction patterns that went to a resolution of 4.2 Å.

Jaffe and Glaeser [1982] developed a "double carbon layer" method which has been applied to PM with a 3.2 Å resolution in electron diffraction; to gap junctions [Unwin, private communication] and to catalase with some
success. The principle advantages of this method are that it does not require any technology other than that included in the standard repertoire of most electron microscopists. The greatest area of improvement over previous methods is that it allows for the controlled evaporation of solvent from the specimens. Assuming that there is a critical time interval $t_c$ in which specimen preservation may be accomplished, $t_c$ is greater for slowly evaporating solvent. This would increase the probability of success. Unlike the stearic acid monolayer method, there is really no special dependence of the technique upon the pH.

There is a possible problem in using the double carbon film technique for specimens that are very sensitive to ionic strength. This is because as one waits for the lens of water to evaporate, the solutes are concentrating. This concentration may be $10^3$ to $10^4$ fold over the initial conditions, leading to possible alterations in the drying environment or even precipitation of solutes.

Among the various methods discussed, the frozen hydration method seems to be the best one in terms of high contrast, high resolution preservation, specimen hydration, and radiation resistance.
1.5.3. Negative staining technique

In addition to the above mentioned methods, a rather common one that can be used even at room temperature is to administer uranyl acetate or phosphotungstic acid (PTA) to a specimen.

In this technique it is commonly thought that the primary purpose of the negative stain is to produce contrast in the image. While negative stains composed of atoms of high atomic number (heavy atoms) do increase the contrast, their most important function may instead be to "support" the structure during drying. Catalase can be used as an example. When dried without the use of a negative stain there is no periodic structure preserved as is evidenced by the complete loss of electron diffraction intensities. When dried in the presence of a negative stain such as uranyl acetate, electron diffraction intensities, in rare cases, can be seen to extend to 8 Å [Glaeser, 1971].

The preservation of structure using a heavy atom stain is not achieved without paying a price. Because of the much higher scattering power of the heavy atoms relative to the atoms making up the biological material, their contribution to the image contrast will be much higher than that of the organic material. Thus the image must be interpreted in terms of the distribution of the stain rather than the structure of the biological specimen.
Unwin [1974] suggested that for negatively stained specimens, there are two distinct stages of irradiation: (a) an initial "fixation" stage (dose < 6 e/Å²) involving considerable atomic rearrangement and associated structural alterations in both stain and protein, and (b) a stain redistribution stage (doses 6 to 600 e/Å²) which he observed, and in which the protein is in a more or less stable condition but the stain continues to alter.

For the last part of my work, I have developed a method where the specimen was first negatively stained, air dried, and frozen in liquid nitrogen, and then transferred to the high resolution cold stage via a cryo-transfer device [Taylor and Glaeser; 1975]. This method has been termed the "NSFT" method. Low dose, low temperature electron micrographs have given 5.7 Å image resolution, as determined from the computer processed Fourier analysis.

In the "NSFT" method it seems possible that both protein and stain contribute to the image contrast and interpretation of images can be difficult. For further discussion please see chapter 5.

1.6. Approaches to high resolution study of HP-protein

For the first part of my work, it was necessary to isolate large, single arrays of HP-protein with good crystalline order. I have indeed obtained large, single
arrays of HP-protein in the size of 15-20 square microns. Chapter 2 describes the methods and results.

For the second part of my work, I have done low dose electron microscopy with the frozen hydrated specimen of crystalline arrays of HP-protein. I have identified measurable structure factors out to 6.9 Å in the computer processed Fourier transform. Details of this piece of work are given in Chapter 3.

A "NSFT" method was used as one of the specimen preparation methods for low dose imaging of the large crystalline arrays of HP-protein. The images have been subjected to image processing procedures, using computer programs developed in this laboratory. Structure factors out to 5.7 Å have been identified. Chapter 4 describes the methods and results of the electron microscopy and image processing of the large arrays of HP-protein, prepared by the NSFT method.

There is now no doubt that high resolution structural features of this protein can be retrieved and eventually a 3-dimensional high resolution model will be obtained to answer a lot of questions about the function and structure of the surface layer protein.
CHAPTER  2

RECONSTITUTION OF LARGE CRYSTALLINE ARRAYS OF HP-PROTEIN

As discussed in chapter 1, in order to do high resolution structural studies on the surface layer protein of *Aquaspirillum serpens*, it is necessary to first obtain large, single arrays with good crystalline order. In this chapter I present the methods and results of reconstitution of large crystalline arrays of HP-protein.

2.1. Methods of specimen preparation for electron microscopy

Electron microscopy was used to monitor the procedures for reconstituting large arrays of HP-protein. In this chapter all the specimens examined in the electron microscope, as described below, were prepared by negative staining with neutralized 1.5% potassium phosphotungstic acid (Sigma, p-4006). Carbon-coated formvar grids were glow discharged to ensure hydrophilicity. Right after the grid was glow discharged, 15 microliters of sample was spread and left on the grids for about 5 min. 15 microliters of stain was added on to the grid for about 1 min. The drop of mixture was then drawn off by a torn piece of filter paper, until only a very thin layer of solution was left on the grid.
2.2. Culture and medium

*Aquaspirillum serpens* strain VHA was initially supplied by Dr. R. G. E. Murray (Department of Bacteriology and Immunology, University of Western Ontario, London, Ontario, N6A5C1, Canada). He suggested a modified Y.P.A. medium for growing the cell strain. The modified Y.P.A. medium was prepared as follows:

First a solution consisting of 0.1% Bacto peptone (Difco, 0118-01-8, control 688846), 0.1% Bacto yeast extract (Difco, 0127-01, control 621856), 0.05% anhydrous sodium acetate (Mallinckrodt, analytical reagent, 7372), 0.025% MgSO$_4$·7H$_2$O (Mallinckrodt, analytical reagent, 6066), and 0.025% anhydrous calcium chloride (Baker and Adamson, reagent 1500) was prepared. The solution was adjusted to pH 7.3 with NaOH and then autoclaved.

Anhydrous L-cystine (Sigma C-8755) was dissolved in basic solution, pH=9, and then injected into the above autoclaved solution, to a final concentration of 0.005%, through a 0.45 micron filter unit (Millex-HA). This method was taken to avoid breaking down of L-cystine during autoclaving. The final pH value of the medium, when measured, was about 7.6.

Cells were maintained and stored at room temperature in test tubes on solid medium, which contained 1.5% agar and the modified Y.P.A. medium.
2.3. Cultivation and harvesting

The method of cell cultivation and harvesting followed the procedures used originally in Murray's lab. [Buckmire and Murray, 1970; Koval and Murray, 1980]

Cells for an initial inoculum and subsequent culture were transferred from the solid medium to 100 ml of a liquid modified Y.P.A. medium. The starter culture was incubated at 37°C with shaking for 48 hr. Cells were then grown in 5-liter flasks, each containing 1 liter of the liquid medium, from an inoculum of 10 ml of the 48 hr starter culture. Flasks were incubated at 37°C on a rotary shaker at 150 rpm for about 16 h. Cells grown by this procedure are shown in Fig. 2-1.

Cells were harvested from the medium by centrifugation (Beckman, Model J-21C, rotor JA-10) at 20,000 x g for 10 min. After removal of the supernatant the cells were washed 4 times by successive suspension in 0.5 mM TRIS (hydroxymethyl)aminomethane buffer (Sigma, reagent grade, No. T-1503), pH 7.4, containing 5 mM calcium chloride (TRIS-Ca²⁺ buffer), and centrifugation (Beckman, Model J-21C, rotor JA-10) at 2500 x g for 10 min at 4°C. Between centrifugations the cells were homogenized with a Thomas tissue homogenizer by using a Teflon pestle. This procedure was used to remove flagella from the cells.
Fig. 2-1. Micrograph of a suspension of *Aquaspirillum serpens* taken with the electron microscope. The specimen was negatively stained with potassium phosphotungstic acid.
2.4. Isolation of outer wall vesicles

The cells with flagella removed were treated in two slightly different ways, referred to in the following as Method A and Method B.

In Method A, the cells with flagella removed were suspended in the TRIS-Ca\textsuperscript{2+} buffer and heated at 60°C for 75 min.\textsuperscript{2} The cells were then centrifuged (Sorvall, RC2-B, SS-34 Rotor) 3 times at 2500 X g for 10 min to remove whole cells and retain the outer wall fragments. The pooled supernatant was then centrifuged (Beckman, L5-75 Ultracentrifuge, 70TI Rotor) at 48,000 X g for 30 min to sediment the outer wall fragments. Examples of the outer wall fragments obtained after centrifugation, labeled sample 1, are seen in Fig. 2-2. Method A was first employed by Buckmire and Murray [1970].

In Method B, the cells with flagella removed were suspended in TRIS-Ca\textsuperscript{2+} buffer and heated at 60°C for 4 hr. Cells were then centrifuged (Sorvall, RC2-B, SS-B) 5 times at 1500 x g for 10 min. Each time the supernatant was saved for recentrifugation. The pooled supernatant was centrifuged (Sorvall RC2-B, SS-34) at 10,000 x g for 10 min and the pellet was resuspended in TRIS-Ca\textsuperscript{2+} buffer.

\textsuperscript{1}In the following discussion the term "outer wall" refers to the complex of outer membrane and surface layer protein.

\textsuperscript{2}the TRIS-Ca\textsuperscript{2+} buffer was used to suspend all samples in the following procedures except otherwise specified.
Fig. 2-2. Micrograph of Sample 1, the outer wall fragments of *Aquaspirillum serpens*, obtained by following the procedure of Buckmire and Murray [1970], which is referred to as Method A in the text. "Naked tubes" are quite frequent. The sample is negatively stained with potassium phosphotungstate.
Examples of the outer wall fragments obtained after centrifugation, labeled sample 2, are seen in Fig. 2-3.

Comparing sample 1 and 2, we see that in sample 2, patches with structured arrays are bigger and more frequent, whereas in sample 1 we see mainly tubes. The yield of Method B was about 10 times that of Method A, judged from the estimated volume of the respective pellets. Method B was thus the preferred method for isolation of outer wall vesicles.

2.5. Formation of large arrays of HP-protein

The abundant outer wall fragments in Sample 2, obtained as described above, were manipulated to form large arrays as follows:

Sample 2 was heated for 7 hr at 60°C in the TRIS-Ca$^{2+}$ buffer. The sample was then labeled Sample 2H and examined right after heating (see Fig. 2-4). We can see a very well ordered, electron transparent (light) array, whereas the dark arrays are oftentime less well ordered and also show Moire patterns. Sample 2H was subsequently stored at 4°C for 2 weeks and examined again. The sample after prolonged storage was labelled 2HS and examined (see Fig. 2-5). At this point the sample contained enlarged, well ordered, light arrays with outer membrane fragments attached to the array in a non-uniform, random pattern.

Observing Sample 2H and Sample 2HS, I developed the
Fig. 2-3. Micrograph of sample 2, the outer wall fragments of *Aquaspirillum serpens*, obtained by "Method B", in which the cells were heated for a longer period, and then centrifuged at lower speed, as compared with Method A. Patches with structured arrays are bigger and more frequent. The yield is increased about 10 times.
Fig. 2-4. Micrograph of Sample 2H, obtained by heating Sample 2 at 60° for 7 hr in the TRIS-Ca\textsuperscript{2+} buffer. The sample was examined right after heating. One can see a very well ordered, electron transparent (light) array about in the center of the micrograph, and dark arrays that are oftentimes less well ordered and also show Moire patterns.
Fig. 2-5. Micrograph of Sample 2HS, obtained by storing sample 2H at 4°C for 2 weeks. Sample 2HS contains enlarged, well ordered, light arrays with outer membrane fragments attached to the array in a non-uniform, random pattern.
postulate that the dark patch was a composite of protein layer and outer membrane. Heating, among doing other things, would separate protein layers and/or molecules from the outer membrane; the released protein layer and/or molecules would build up into enlarged crystalline arrays during prolonged storage; meanwhile, outer membrane fragments could attach to the protein array.

I have also tried many other possible ways of heating, centrifugation and storage. Procedures below are very illustrative:

Sample 2, obtained as described above, was heated for 7 hr and centrifuged (Sorvall, RC2-B, SS-34 Rotor) at 1500 X g for 10 min. The pellet and supernatant were labeled sample 3 and 4, respectively. Sample 3 (the pellet of the first centrifugation after 7 hr heating) was stored at 4°C and examined after 3 weeks (see Fig. 2-6). Sample 4 (the supernatant of the first centrifugation after 7 hr heating) was examined right after heating (see Fig. 2-7), and then centrifuged at 2500 X g for 10 min (Sorvall, RC2-B, SS-34 Rotor). The pellet and supernatant of this second centrifugation were labeled Sample 5 and 6, respectively. Sample 5 was stored at 4°C and examined after 3 weeks (see Fig. 2-8). Sample 6 also was stored at 4°C and examined after 3 weeks (see Fig. 2-9). To sum up, sample 3 (pellet of the first centrifugation after 7 hr heating), Sample 5 (pellet of the second centrifugation after 7 hr
Fig. 2-6. Micrograph of Sample 3, which is the pellet of the first centrifugation after 7 hr heating of Sample 2, examined after 3 weeks of storage. Sample 3 contains large dark patches.
Fig. 2-7. Micrograph of Sample 4, which is the supernatant of the first centrifugation after 7 hr heating of Sample 2, examined right after heating. One can see small light arrays as well as outer membrane fragments.
Fig. 2-8. Micrograph of Sample 5, which is the pellet of the second centrifugation after 7 hr heating of Sample 2, examined after 3 weeks of storage. One can see very large light arrays of HP-protein. The center to center distance is 145 Å.
Fig. 2-9. Micrograph of Sample 6, which is the supernatant of the second centrifugation after 7 hr heating of Sample 2, examined after 3 weeks of storage. One can see more frequent naked patches, which are outer membrane fragments or their derivatives.
heating) and Sample 6 (supernatant of the second centrifugation after 7 hr heating) were all stored for 3 weeks and examined (see Fig. 2-6, 8, 9); whereas Sample 4 (supernatant of the first centrifugation after 7 hr heating) was examined right after 7 hr heating (see Fig. 2-7). Fig. 2-10 summarizes the procedures.

Sample 3 had large dark patches, but did not show large light arrays. Sample 5 had tremendously well ordered large, light arrays. Sample 6 had more frequent naked patches, which were outer membrane fragments or their derivatives. A plausible theory is that sample 5 contained relatively more of the small protein arrays, and relatively less outer membrane fragments, small or large. The small protein arrays subsequently built up into large arrays during storage. Sample 3 might be rich in large outer membrane fragments; whereas sample 6 was low in protein arrays but rich in outer membrane fragments.

Dr. R. M. Glaeser suggested an alternative model for crystal formation (personal communication). The details of Dr. Glaeser's model is presented in chapter 5, section 5.1.

I have also discovered that with the light, well ordered array, regardless of its age and size, the optical diffraction pattern (see Fig. 2-11) always shows "handedness" (i.e., absence of mirror symmetry). Whereas with the dark patches, the optical diffraction patterns appear
Fig. 2-10. A flow chart of the procedure for examining Sample 3, 4, 5 and 6.
SAMPLE 2

Heated at 60°C in TRIS-Ca⁺⁺ buffer for 7 hr
Centrifuged at 1500 X g for 10 min

SAMPLE 3
(pellet)

SAMPLE 4
(supernatant)

examined right away (see Fig. 2-7)

Centrifuged at 2500 X g for 10 min

SAMPLE 5
(pellet)

SAMPLE 6
(supernatant)

Stored for 3 wks
Examined
(see Fig. 2-6)

Stored for 3 wks
Examined
(see Fig. 2-8)

Stored for 3 wks
Examined
(see Fig. 2-9)
Fig. 2-11. The optical diffraction pattern of light, well ordered arrays. "Handedness" (i.e., absence of mirror symmetry) is obvious.
to be either a single, symmetrical pattern (see Fig. 2-12a), or composite, symmetrical patterns (see Fig. 2-12b,c,d); indicating they are really closed, collapsed vesicles with protein layers on both sides which may or may not be in close register to each other.

The procedure of obtaining large arrays, the appearance of light and dark arrays, and the different optical diffraction patterns of light and dark arrays all point to the conclusion that these light arrays are real single layer protein arrays, whereas the dark arrays dealt with in previous work were closed outer wall vesicles with 2 layers of protein, collapsed on the grid.

The large, light arrays obtained as described above seemed to be extremely well ordered. Low dose low temperature electron microscopy was then applied for high resolution study of HP-protein.
Fig. 2-12a. The optical diffraction pattern of the dark patches, which is a single, symmetrical pattern in this case.
Fig. 2-12b. The optical diffraction pattern of the dark patch, which is a composite, symmetrical pattern in this case.
Fig. 2-12c. The optical diffraction pattern of the dark patch, which is a composite, symmetrical pattern in this case.
Fig. 2-12d. The optical diffraction pattern of the dark patch, which is a composite, symmetrical pattern in this case.
CHAPTER 3

STRUCTURAL ANALYSIS OF FROZEN HYDRATED SPECIMENS OF HP-PROTEIN

As discussed in chapter 1, the frozen hydrated specimen method is probably the best method for electron microscopy, from the overall consideration of specimen hydration, high resolution structure preservation, radiation resistance and contrast enhancement.

In this chapter I present the procedure and result of structural analysis of frozen hydrated specimens of HP-protein.

3.1. Sample preparation of frozen-hydrated specimens

The method of "double carbon layer" [Jaffe, Ph.D. thesis, 1982] was employed with some modifications.

Carbon was evaporated onto freshly cleaved mica. Small pieces of mica can be placed at variable distances from the carbon electrodes. Those which are closer to the source will have more carbon evaporated onto them and the ones further away will have less. Using this methodology, it was quite easy to achieve a range of carbon thickness which subsequently led to good specimen preservation.

Copper grids were placed on filter paper at the bottom of a dish full of distilled water. A carbon film obtained as described above was floated off onto the water surface in the dish. Water was sucked off carefully until
the carbon film rested on the grids at the bottom. The complex of filter paper, grids and carbon film was air-dried overnight. A large number of carbon coated grids could thus be prepared beforehand. Carbon coated grids prepared in this way also seemed to have less holes and breaks.

Five microliters of "sample 5", obtained as described in chapter 2, was applied and left on a carbon coated grid at room temperature for 15 min. This long a period of time was used in order to get good coverage of the grid with protein arrays. The grid was then placed back down into a dish of distilled water and the sample solution was washed off before bringing the grid up through the carbon film for the second layer. The result of this procedure was to sandwich the specimen between two layers of carbon films. Only grids that were found to have a "lens" of water on both sides were used for further experimental work. At first this lens of water was continuous between both sides of the grid, however, after some evaporation the rim of the grid would divide the lens into two, one on each side. It was then possible to blot off the water on the back side of the grid with filter paper. It was then necessary to wait further, until the remaining lens slowly evaporated, rendering the specimen a purplish-orange color. Throughout this process the grid was monitored with an ordinary incandescent bulb placed around 8 inches
from the object. A shiny surface of the grid was always indicative of a layer of water, that was found to be too thick upon freezing. Care should be taken not to air dry the specimen, however. When the specimen was judged to be of the correct thickness it was then inserted into LN$_2$.

3.2. Electron microscopy of frozen hydrated HP-protein

After freezing in LN$_2$ the specimen was transferred via a cryotransfer device [Taylor and Glaeser; 1975], to the high resolution cold stage [Hayward and Glaeser, 1980], precooled to thermal equilibrium (around $-120^\circ$C), on a JEOL 100B microscope.

Two devices were used in conjunction with the microscope: a solid state detector and a remote control "Blue Box". The solid state detector was used to monitor the dose rate of electron exposures received by the specimen. The remote control "Blue Box", an electronic device that applies a variable external current to the second condenser (C$_2$) lens, allowed setting the dose rate wanted for scanning, say, on channel #1, as well as the dose rate wanted for taking images, say, on channel #2. The length of the exposure time can also be set on channel #2, for taking images. A "shutter" on the remote control deflects the beam within the electron gun, thus preventing any part of the specimen from being irradiated when it is switched on.
Before scanning the grid, the beam illumination conditions were set up for different modes of operation. Let it be noted that the following described operations were all done at 40,000 X, except otherwise specified. First of all the beam was adjusted to be about 12 cm in diameter on the final viewing screen, through adjustment of the current on channel #2 of the remote control, and the beam was centered at the middle of the normal viewing screen, using the normal bright-field beam-alignment controls. The beam was then condensed to be about 1 cm in diameter, by the normal control on the microscope, and the beam was centered at the middle of a second fluorescent screen, mounted in front of the standard one, using the alternate (dark field) deflection coil of the microscope.

The grid was scanned with a dose rate of 1 electron/Å²-min, adjusted through channel #1 of the remote control. The specimen was observed with the microscope in the "selected area diffraction mode" but with a highly defocused diffraction spot. When promising areas of specimens were found in the field of view, the desired crystal was brought to the center of the screen. The shutter on the remote control was switched on, to shut off the beam. Alignment control was then switched to the alternate (dark field) deflection coil, using the control on the microscope, and the C2 control was switched to the normal microscope control. The shutter was then switched
off and a condensed beam appeared on the front screen for focusing. This was done to avoid premature exposure of the desired crystal area by the beam.

Focusing was accomplished on the front screen, using the phase contrast granularity of the support film as the criterion for correct focus. About 3000 to 5000 Å under-focus\(^1\) was set by turning the focusing knobs away from the "in focus" condition by an amount determined from focal series studies, done beforehand.

After the focus was correctly adjusted, the shutter on the remote control was switched on again, to shut off the beam. The control on the microscope was switched to bright field and channel #2 of the remote control was connected. The specimen was then photographed by raising the camera shutter, and switching off the shutter on the remote control. The dose rate for imaging was preset to be about 8 electrons/Å\(^2\)-sec, and the exposure time was preset to be 0.5 sec. The resultant exposure was then about 4 electrons/Å\(^2\). A second image was then taken at about 2 micrometers underfocus. Kodak Electron Image Film SO-163 was used for recording images, and the films were developed in full strength D-19 for 12 min.

\(^1\)underfocus refers to a lens current smaller than the lens current for the "in-focus" condition. In the under-focus condition, defocus "off-sets" the effect of spherical aberration, i.e., \(\Delta z\) is positive in the equation for the contrast transfer function defined in chapter 1.
3.3. Image processing

Fig. 3-1, 3-2 and 3-3 show examples of images, taken at about 2 micrometer underfocus, of the frozen hydrated specimen of the HP-protein. Fig. 3-2 shows a large sheet of HP-protein, with an additional "thick object" (perhaps a lysed cell) sitting on top of it. As shown in the boxed-off area of Fig. 3-2, especially good side views of HP-protein are recorded. In Fig. 3-3 the optical diffraction pattern of the single-layered area in the boxed-off area is shown in the insert. The "handedness" of the pattern is clearly seen.

Images were screened for further processing by optical diffraction. Once the position of a good area was located approximately on the second, highly defocused image, the corresponding area on the first image was scanned carefully to select a good area for further processing. A selected area was scanned on a Perkin-Elmer scanning microdensitometer with a sampling distance corresponding to 3.5 Å at the specimen.

Most programs used for image processing were part of the system written at Lawrence Berkeley Laboratory by Dr. D. Grano. Some additional programs were written as needed for processing this image of frozen hydrated specimen.

Digitized arrays of 512 X 512 picture elements were Fourier transformed by means of the Fast Fourier Transform (FFT) algorithm. Fig. 3-4 shows a display of the
Fig. 3-1. An image of a frozen-hydrated specimen of HP-protein, taken at about 2 micrometer underfocus.
Fig. 3-2. An image of a frozen-hydrated specimen of HP-protein, taken at about 2 micrometer underfocus. This is a large sheet of HP-protein, with an additional "thick object" (perhaps a "lysed cell") sitting on top of it. In the boxed-off area is shown especially good side views of HP-protein.
Fig. 3-3. An image of a frozen hydrated specimen of HP-protein. The optical diffraction pattern of the single layered array in the boxed off area is shown in the insert. The center to center distance is 145 Å.
Fig. 3-4. A display of the logarithm of the power spectrum of the discrete Fourier transform of a scanned area, for the case of the frozen hydrated specimen of HP-protein.
logarithm of the power spectrum of the discrete Fourier transform. Fig. 3-5 is another display of the same transform, over exposed in order to reveal the phase contrast transfer function.

The output of this Fourier transform was also displayed graphically on computer hard copy output with numbers of columns and rows shown, so that some of the strong diffraction spots at lower resolution could be located. The positions of these spots were then fitted to a lattice by a least-square procedure, using the computer program which is called "Lattice Look". The output of the Lattice Look program consists of 9 X 9 blocks of amplitudes and phases. Fig. 3-6 shows an example of the output blocks. Those blocks were used to locate the strong reflections accurately, and these locations were again fitted to a lattice by a least-square procedure. This lattice locating procedure was repeated until the positions of the least-square lattice points shifted not more than one data point. All amplitude peaks within one data point of these lattice locations were noted, along with the corresponding phases.

From Fig. 3-5, one can locate the first zero of the contrast transfer function. A program was written to estimate the value of underfocus by using the following equation:
Fig. 3-5. Another display of the same transform as in Fig. 3-4, over exposed in order to reveal the phase contrast transfer function.
Fig. 3-6. An example of a output block of the lattice look program. The sample dealt with was prepared by the frozen hydrated method.
H = -1  K = 1

LOCATION OF LARGEST PEAK IN BLOCK = (245, 264)

EXPECTED LOCATION = (244.933, 263.915)

LOCATION ERROR - ASTAR COMP= 0.006, BSTAR COMP= 0.003

AMPLITUDE = 0.7500E+05  PHASE = 272.23

AVERAGE AMPLITUDE = 0.1458E+05

SCALE= 0.1E-01  MIDDLE=(245, 264)

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\[
\Delta z = C_s \lambda^2 \frac{s^2}{2} - n \lambda s^2,
\]
where \( n = -1 \) for the first zero of the contrast transfer function; \( C_s = 1.4 \) mm (Dr. G. Cogswell, Vice President of the JEOL company, personal communication); \( \lambda = 0.037 \) \( \text{\AA} \).

Astigmatism was obvious, as seen in Fig. 3-5. In the following step, values of underfocus were determined with finite width, due to an uncertainty in the positions of the first zero. Along the long axis of the ellipse defined by the first zeros of the contrast transfer function, the underfocus \( (\Delta z_L) \) was determined to be between 3280 \( (\Delta z_{L1}) \) and 3390 \( (\Delta z_{L2}) \) \( \text{\AA} \), while along the short axis the underfocus \( (\Delta z_S) \) was between 4640 \( (\Delta z_{S1}) \) and 4850 \( (\Delta z_{S2}) \) \( \text{\AA} \). The program written to determine the value of underfocus would also plot out the contrast transfer function (CTF) by using the following equation:

\[
\text{CTF}(s) = \sin \left( 2\pi \left( C_s \lambda^3 s^4/4 - \Delta z \lambda s^2/2 \right) \right)
\]

Fig. 3-7 is an example of two contrast transfer functions, shown as envelopes corresponding to the range of uncertainty in \( \Delta z \), one along the long axis of the ellipse defined by the first zero of the contrast transfer function, the other along the short axis. One can see that, at higher resolutions, the two functions can be shifted with respect to each other and have opposite sign. Needless to say, this was a rough estimation. For frequencies larger than the frequency of the first zero of a contrast
Fig. 3-7. An example of two contrast transfer functions, shown as envelopes corresponding to the range of uncertainty in $\Delta z$, one along the long axis of the ellipse defined by the first zero of the contrast transfer function ($\sin \gamma_{L1}$ and $\sin \gamma_{L2}$), as shown in Fig. 3-5, the other along the short axis ($\sin \gamma_{S1}$ and $\sin \gamma_{S2}$).
transfer function, the sign of the function near to its zeros has a considerable degree of uncertainty. Four especially strong triplets were identified from the output of the Lattice Look program. Phase correction was carried out, if necessary, to account for a difference in sign of the contrast transfer function for symmetry related reflections. These 4 triplets were used in the "phase origin refinement" program to search for one of the 6-fold symmetry axes as a phase origin. Table 3-1 shows the result of such a phase origin search. The phase shift applied was 321.0° for (1, 0) and 223.9° for (0, 1). The RMS phase error associated with this origin was 10.9 degree.

A program was then written to list the value of phase shift, defined as below, of all the structure factors, given the phase shift determined for (1, 0) and (0, 1) by the above phase origin search program, i.e.,

\[ \text{phase shift} = (h \times 321.0 + k \times 223.9) \]

for a given reflection of Miller index (h, k).

A statistical analysis was done to determine the existence of peaks above a local background. All amplitude peaks within one data point of lattice locations were noted from the output of the lattice look program. The output of the lattice look program also listed the averaged amplitude of each output block (see Fig. 3-6), Let A
Table 3-1. The output of the "phase origin refinement" program. Four triplets are used in the program to search for one of the 6-fold symmetry axes as a phase origin.
THE INPUT DATA WILL BE DISPLAYED WITH RECALCULATED PHASES AND ERRORS
TODAY'S DATE - 22-JUL-83 TIME - 13:11:50
COMMENTS - test

PHASE SHIFT APPLIED - 1,0 = 321.00 0,1 = 223.90

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<th>NEW PHS</th>
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THE RMS PHASE ERROR ASSOCIATED WITH THIS ORIGIN = 10.90
denote the average of the 81 data values in the output block (see Fig. 3-6); $A_p$ denote the value of the amplitude peak, $A'$ denotes the average of the 80 data values, i.e., excluding the amplitude peak; and s.d. denote the background standard deviation, i.e., standard deviation of the 80 data values, excluding the amplitude peak. Then, it is easy to see that:

$$A' = \frac{(81 A - A_p)}{80}$$

From s.d. = $A'/2$ [Hayward and Stroud, 1981], $A_{s.d.}$ (the value of amplitude peak expressed in units of background-standard-deviation above the average background) can be derived:

$$A_{s.d.} = \frac{(A_p - A')}{s.d.}$$

$$= \frac{(A_p - A')}{(A'/2)}$$

$$= 2 \left[ \frac{A_p}{A'} - 1 \right]$$

$$= 2 \left\{ \frac{A_p}{\left[ \frac{(81A - A_p)}{80} \right]} - 1 \right\}$$

$$= 2 \left\{ \frac{80}{\left[ 81 \left( 1/(A_p/A) \right) - 1 \right]} - 1 \right\}$$

The functional relationship of $A_{s.d.}$ to $A_p/A$ is shown in Fig. 3-8. Peaks greater than 3 standard deviations above the background were identified up to 13.70 Å resolution, which was below the limit of the first zero of the contrast transfer function in all directions.

For those spots identified as peaks, the values of $m$ were calculated, where
"A_s,d." denotes the amplitude, expressed in units of background standard deviations, above the average background. "A" denotes the average of the 81 data values in the output block, as shown in Fig. 3-6. "A_p" denote the value of the amplitude peak.
\[ m = \cos \theta = \cos (\text{old phase} + \text{phase shift}) \]

where "old phase" is the experimental result as shown in the output of the lattice look program (see Fig. 3-6), and "phase shift" is equal to \((h \times 321.0 + k \times 223.9)\), as mentioned above; and \(\theta (= \text{old phase} + \text{phase shift})\) should be close to 0 or 180, aside from an integral multiple of 360. Table 3-2 lists the amplitudes, in units of standard deviation above the average background \((A_{s.d.})\); values of \(m(=\cos \theta)\); amplitudes, averaged among the symmetry related reflections; and phases of peaks so identified, up to 13.70 Å.

An inverse Fourier transform was applied to the amplitudes and phases of those reflections, shown in Table 3-2, to obtain the averaged real space image, up to a resolution of 13.70 Å. An example of such a computer processed image is shown in Fig. 3-9. Discussion will be delayed until a higher resolution map is presented as below.

Attention was then focused on the domain beyond 13.70 Å. At higher resolution, the contrast transfer function begins to oscillate frequently. Two values of the contrast transfer function, \(\sin \gamma_1\) and \(\sin \gamma_2\), were obtained for each reciprocal lattice point, due to an uncertainty in \(\Delta z\), as explained above. Those spatial frequencies for which \(\sin \gamma_1\) and \(\sin \gamma_2\) have opposite sign were not taken
Table 3-2. A list of peaks identified for the case of frozen hydrated method, up to 13.70 Å. "A_{s.d.}" denotes amplitude, in units of background standard deviations, above the average background; "m" is defined as \( \cos \theta \), where \( \theta \) is the old phase + phase shift; \( \text{ampl.}(A_p) \) is the amplitude of the peak that is located within one data point of the exact lattice location.
### The Frozen Hydrated Method

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Fig. 3-9. Computer processed image of the specimen prepared by the frozen hydrated method, up to a resolution of 13.70 Å.
into consideration. All amplitude peaks within one data point of the lattice locations, determined with the lattice look program as described above, were noted, along with the corresponding phases. Peaks with amplitudes that were 2.8 standard-deviation above the average background were included. Peaks that were singlets, i.e., only one of the symmetry related reflections had a peak within one data point of the lattice location, were not considered. Using these criteria, 1 triplet and 1 doublet were identified to be valid reflections as shown in Table 3-3. From the sign of \( \cos \theta \) and the sign of \( \sin \gamma_1 \) and \( \sin \gamma_2 \), phases can be assigned to those peaks consistently.

It was most interesting to note that for the doublet \((5, 15)\) and \((15, -20)\), the signs for the 2 associated contrast transfer functions were different. A program was written to plot out the 2 transfer functions, as shown in Fig. 3-10. It can be seen that a larger underfocus value associated with the direction of the \((5, 15)\) reflection results in a faster oscillating \( \sin \gamma \), and \((5, 15)\) was located in the zone between the third and forth zeroes; whereas \((15, -20)\) was located in the zone between the second and third zeroes. The sign of \( \sin \gamma \) associated with \((5, 15)\) was therefore positive whereas that with \((15, -20)\) was negative.

Many singlets were identifiable in the output of the lattice look program. However, they were not included
Table 3-3. A list of peaks identified beyond 13.70 Å. Two values of the contrast transfer function, \( \sin \gamma_1 \) and \( \sin \gamma_2 \) are obtained for each reciprocal lattice point, due to an uncertainty in \( \Delta z \). The spatial frequencies for which \( \sin \gamma_1 \) and \( \sin \gamma_2 \) have opposite sign are not included. All amplitude peaks within one data point of the lattice locations were noted, along with the corresponding phases. Peaks that are singlets are not considered. Peaks with \( A_{s.d.} > 2.8 \) are included.
## THE FROZEN HYDRATED METHOD

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Fig. 3-10. Graphs that give the values of Contrast transfer functions, shown as envelopes ($\sin\gamma_1$ and $\sin\gamma_2$) to account for the uncertainty in the values of under-focus, of the two reflections with Miller index of (5, 15) and (15, -20) respectively.
graphs giving values of CTF of the reflection: (15, -20) \( \sin \gamma_1 \), \( \sin \gamma_2 \)

6.966 Å

graphs giving values of CTF of the reflection: (5, 15) \( \sin \gamma_1 \), \( \sin \gamma_2 \)

20 0.05

10 0.10

5.0 d (Å)

6.67 0.15

6.67 0.15

5.0 d (Å)

XBL837-3842
because the consistency of phases within symmetry related reflections could not be checked.

An inverse Fourier transform was applied to the amplitudes and phases of all the peaks identified, i.e., peaks below and above 13.70 Å, to obtain an averaged real space image, that includes the limited data at a resolution of 6.9 Å, as shown in Fig. 3-11.

Similar new features can be noted between the maps shown in Fig. 3-9 and 3-11 as follows: the overall appearance of contrast variation coincide with each other; the handedness of both images is quite evident; a white contrast feature is present at the six-fold lattice positions; a new connection between all adjacent morphological units emerges across the two-fold axis between morphological units (hexamers); details of the protein-protein contact at the three-fold lattice position are beginning to emerge.

The map in Fig. 3-11 has finer variation than in Fig. 3-9, as expected. Around the 6-fold lattice position, a faint six-angle "star" can be seen in Fig. 3-11.

It should be bourn in mind that both maps shown in Fig. 3-9 and 3-11 are incomplete representations of the full structure. First of all, the crystal is small, not very well ordered, and located at the rim of the screen (see Fig. 3-3). The criterion that Fourier coefficients should be 3 standard deviations above the average
Fig. 3-11. A computer processed image of the specimen prepared by the frozen hydrated method up to 6.966 Å obtained by applying an inverse Fourier transform to all the peaks identified, as shown in Table 3-2 and Table 3-3.
background disqualifies many potential peaks, in the case for building the 13.70 Å map. As for the 6.9 Å map, the fast oscillating contrast transfer function, the uncertainty in Δx, as well as the exclusion of singlets, all make the map very incomplete. For more discussion, please see chapter 4.
STRUCTURAL ANALYSIS OF HP-PROTEIN PREPARED BY THE NSFT METHOD

As discussed in chapter 1, air drying a specimen with negative stain may still render the specimen hydrated, or partly hydrated. Negatively stained catalase can be preserved to 8 Å resolution, as seen by electron diffraction [Glaeser, 1971]; and negatively stained stacked disk aggregates of tobacco mosaic virus protein can be preserved to 12 Å image resolution [Unwin, 1974]. An "NSFT" method was developed for imaging the large arrays of HP-protein, obtained as described in chapter 2. The methods and results are given below.

4.1. The NSFT method

As introduced in chapter 1, the "NSFT" method refers to the procedure as follows:

1. The specimen was negatively stained with potassium phosphotungstic acid in the same way as described in the beginning of chapter 2, except the amount of stain applied was reduced to 5 microliters. The stain was very carefully neutralized beforehand. The grid was air-dried for 15 min.

2. The specimen was then inserted into liquid nitrogen.

3. After freezing in liquid nitrogen the specimen was transferred to the high resolution cold stage [Hayward and
Glaeser, 1980], in the same way as for the frozen hydrated specimen, described in chapter 3.

4.2. Low dose, low temperature electron microscopy

The procedure of low dose, low temperature electron microscopy of the specimen prepared by the NSFT method was the same as that of the frozen-hydrated specimen. The dose used for imaging was about 8 electrons/\AA^2. For details please see chapter 3.

4.3. Image processing

Fig. 4-1 is an example of an image, taken at low dose and low temperature, of the reconstituted large arrays of HP-protein, prepared by the NSFT method. The optical diffraction pattern is shown in the insert. The "handedness" of the pattern is clearly seen.

Images were screened for further processing by optical diffraction. A selected area was scanned on a Perkin-Elmer scanning microdensitometer with a sampling distance corresponding to 2.5 \AA at the specimen.

Image processing was carried out similar to the way that has been described in chapter 3, except no attempts were made to estimate the contrast transfer function. Fig. 4-2 shows a display of a scanned image. Fig. 4-3 shows a display of the logarithm of the power spectrum of the discrete Fourier transform for this same area.

An example of the output of the lattice look
Fig. 4-1. An image, taken at low dose and low temperature, of the reconstituted large arrays of HP-protein, prepared by the NSFT method. The optical diffraction pattern is shown in the insert. The "handedness" of the pattern is clearly seen. The center to center distance is 145 Å.
Fig. 4-2. A display of a scanned image for the case of NSFT method. The center to center distance is 145 \( R \).
Fig. 4-3. A display of the logarithm of the power spectrum of the discrete Fourier transform of the image shown in Fig. 4-2.
program is shown in Fig. 4-4. Six sets of symmetry-related triplets were used for phase origin refinement and the results are shown in Table 4-1. The phase shifts applied were 224.7° for (1, 0) and 121.4° for (0, 1). The RMS phase error associated with this origin was 6.61°. Values of $m(=\cos \theta)$ were determined in the same way as described in chapter 3.

Peaks greater than 3 s.d. above the average background were identified. Table 4-2 lists $A_{\text{s.d.}}$ (amplitudes in units of background-standard-deviation above the average background, see chapter 3 for definition), and $|m|$ of those peaks up to a resolution of 5.7 Å. The high values of amplitudes ($>3$ s.d.) and $|m|$ (close to 1) suggest that those peaks are truly valid structure factor coefficients.

An inverse Fourier transform was applied to the amplitudes and phases of peaks up to 13.70 Å, which was below where the first zero of the transfer function occurred in any direction. There can be no confusion as regards the sign of the contrast transfer function within this resolution. Using the data for specimens prepared by the NSFT method, as shown in Table 4-3, an averaged real space image, with an image resolution of 13.70 Å, was obtained and is shown in Fig. 4-5.

The computer processed image shown in Fig. 4-5 appears to be fairly different from that shown in Fig. 3-
Fig. 4-4. An example of the output of the lattice look program for the case of NSFT method.
\begin{align*}
H &= 2 \quad K = 2 \\
\text{LOCATION OF LARGEST PEAK IN BLOCK} &= (284, 281) \\
\text{EXPECTED LOCATION} &= (284.007, 280.500) \\
\text{LOCATION ERROR - ASTAR COMP} &= -0.019, \quad \text{BSTAR COMP} = 0.055 \\
\text{AMPLITUDE} &= 0.1866E+06 \quad \text{PHASE} = 210.38 \\
\text{AVERAGE AMPLITUDE} &= 0.2989E+05 \\
\text{SCALE} &= 0.1E-02 \quad \text{MIDDLE} = (284, 280) \\
\end{align*}

\begin{tabular}{cccccccccccccccccccc}
\end{tabular}
Table 4-1. The output of the "phase origin refinement" program in the case of the NSFT method. Six triplets are used in the program to search for one of the 6-fold symmetry axes as a phase origin.
THE INPUT DATA WILL BE DISPLAYED WITH RECALCULATED PHASES AND ERRORS

TODAY'S DATE - 9-JUN-83    TIME - 13:39:47
COMMENTS - 025bb real triplets

PHASE SHIFT APPLIED - 1,0 = 224.70     0,1 = 121.40

<table>
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<th>TRIP#</th>
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<th>NEW_PHS</th>
<th>THEORY</th>
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<td>STD. DEV.</td>
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<td>457000</td>
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<td>0.0</td>
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<td>2</td>
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<td>182.6</td>
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<td>153000</td>
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<td>179.5</td>
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THE RMS PHASE ERROR ASSOCIATED WITH THIS ORIGIN = 6.61
Table 4-2. A list of peaks identified up to 5.7 Å in the case of the NSFT method. Peaks within one data point of the lattice locations and with $A_{\text{s.d.}} > 3$ are included.
## THE NSFT METHOD

| H | K | Bragg spacing (angstroms) | \(A_{s.d.}\) | \(|\cos(\theta)|\) |
|---|---|--------------------------|---------|----------------|
| 1 | 0 | 125.57                   | 31.5    | 1.00           |
| -1| 1 | 125.57                   | 20.5    | 1.00           |
| 0 | -1| 125.57                   | 19.2    | 1.00           |
| 2 | 0 | 62.79                    | 32.2    | 0.99           |
| -2| 2 | 62.79                    | 15.2    | 0.99           |
| 0 | -2| 62.79                    | 25.8    | 0.99           |
| 2 | 1 | 47.46                    | 12.8    | 0.99           |
| -3| 2 | 47.46                    | 11.6    | 0.98           |
| 1 | -3| 47.46                    | 11.0    | 1.00           |
| 3 | 0 | 41.86                    | 8.2     | 0.98           |
| -3| 3 | 41.86                    | 7.0     | 1.00           |
| 0 | -3| 41.86                    | 8.7     | 0.99           |
| 2 | 2 | 36.25                    | 11.1    | 1.00           |
| -4| 2 | 36.25                    | 8.2     | 1.00           |
| 2 | -4| 36.25                    | 6.7     | 1.00           |
| 4 | 0 | 31.39                    | 5.5     | 0.98           |
| -4| 4 | 31.39                    | 8.9     | 0.95           |
| -5| 2 | 28.81                    | 4.8     | 1.00           |
| 3 | -5| 28.81                    | 4.8     | 0.98           |
| 3 | 2 | 28.81                    | 6.4     | 0.97           |
| 2 | -5| 28.81                    | 4.1     | 0.97           |
| 1 | 4 | 27.40                    | 3.6     | 0.71           |
| 5 | 0 | 25.11                    | 4.1     | 0.72           |
| 0 | -5| 25.11                    | 3.0     | 0.99           |
| 4 | -6| 23.73                    | 3.8     | 0.99           |
| 5 | 1 | 22.55                    | 7.0     | 1.00           |
| -6| 5 | 22.55                    | 5.5     | 1.00           |
| 1 | -6| 22.55                    | 5.9     | 0.99           |
| -6| 6 | 20.93                    | 3.1     | 0.97           |
| -7| 4 | 20.64                    | 3.0     | 0.44           |
| 5 | -7| 20.11                    | 3.8     | 0.96           |
| -7| 1 | 19.15                    | 3.1     | 0.87           |
| 4 | 4 | 18.13                    | 4.8     | 0.80           |
| -8| 4 | 18.13                    | 4.1     | 0.42           |
| 6 | -8| 17.41                    | 3.0     | 0.68           |
| 4 | -9| 16.08                    | 3.5     | 0.44           |
| -9| 8 | 14.70                    | 3.4     | 0.67           |
| -10| 8 | 13.70                    | 3.4     | 0.30           |
| 9 | -10| 13.16                   | 3.4     | 0.96           |
| 6 | 5 | 13.16                    | 3.6     | 0.83           |
| -11| 3| 12.75                    | 4.2     | 1.00           |
| -12| 5| 12.03                    | 4.3     | 0.94           |
| -12| 7| 12.03                    | 3.3     | 0.79           |
| 3 | -13| 10.65                   | 3.6     | 0.69           |

(Table 4-2 to be continued.)
| H  | K  | Bragg spacing (angstroms) | A_s.d. | |cos (θ)|
|----|----|--------------------------|--------|--------|
| -16| 8  | 9.06                     | 3.2    | 1.00   |
| 3  | -16| 8.52                     | 3.3    | 0.63   |
| 2  | 14 | 8.32                     | 5.0    | 0.97   |
| -16| 2  | 8.32                     | 4.8    | 0.52   |
| -19| 6  | 7.46                     | 4.2    | 0.88   |
| 1  | 17 | 7.17                     | 3.6    | 1.00   |
| 4  | -20| 6.85                     | 3.6    | 0.69   |
| -21| 7  | 6.78                     | 3.8    | 0.76   |
| 17 | -20| 6.72                     | 4.7    | 0.59   |
| 11 | 11 | 6.59                     | 4.3    | 0.62   |
| 11 | -22| 6.59                     | 3.3    | 0.94   |
| 4  | 17 | 6.50                     | 4.7    | 0.50   |
| 17 | -21| 6.50                     | 3.5    | 0.59   |
| -22| 7  | 6.45                     | 3.9    | 0.70   |
| 19 | -20| 6.43                     | 4.6    | 0.95   |
| -22| 6  | 6.38                     | 3.2    | 0.87   |
| 12 | 11 | 6.30                     | 3.5    | 1.00   |
| -22| 17 | 6.29                     | 3.6    | 0.68   |
| 18 | 4  | 6.19                     | 4.3    | 0.67   |
| 3  | 19 | 6.08                     | 3.3    | 0.86   |
| 22 | 0  | 5.71                     | 3.3    | 0.95   |
Table 4-3. A list of peaks identified up to 13.70 Å in the case of the NSFT method. The criteria are the same as in the case of the frozen hydrated method, as shown in Table 3-2.
### THE NSFT METHOD

**H K Bragg As.d. m ampl. phase spacing (Å)**

```
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<th>As.d. m</th>
<th>ampl. phase</th>
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<td>32.2</td>
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Fig. 4-5. The computer processed image of the specimen prepared by the NSFT method, up to a resolution of 13.70 Å.
9, which was derived from the frozen hydrated specimen. The white contrast feature at the six-fold lattice positions, as shown in Fig. 3-9, does not appear in Fig. 4-5. The connection across the two-fold axis between morphological units, as shown in Fig. 3-9, does not appear in Fig. 4-5. The contrast features around the protein-protein contact at the three-fold lattice position are different for the two cases. The dense portions are not exactly the same for the two cases, either. For further discussion of the results of the frozen hydrated and NSFT method, please see chapter 5.
CHAPTER 5

DISCUSSION AND SUMMARY

As presented in chapter 2, 3, and 4, large arrays of HP-protein were obtained and structural analysis was done by the frozen hydrated and the NSFT method.

Several points are worth discussion, such as: What is the mechanism of crystal formation? How does the protein from outer wall vesicles compare with that from single layer arrays? How do the results of the frozen hydrated method compared with those of the NSFT method? Will it be possible to extend the structure analysis to even higher resolution? Answers to those questions are suggested below:

5.1. Thoughts on mechanism of crystal formation

Dr. R.M. Glaeser suggested a model for crystal formation (personal communication). The attachment of protein molecules to the outer membrane is proposed to trigger a conformational change that propagates to the tips of the flared-out spokes, where the protein-protein contacts occur in the formation of arrays. "Heating" is proposed to induce the same conformational changes that resulted in the formation of arrays. This model can be tested by isolating protein molecules and then thermally treating the sample under the same condition by which the outer wall
counterpart successfully produced large arrays.

The method of isolating protein molecules has already been developed by Buckmire and Murray [1970], and it is outlined below:

The sample of outer wall vesicles, prepared by Method A as described in chapter 2, is suspended in 1.5 M guanidine hydrochloride, pH 7.0. After incubation at room temperature for 2 hr the residual outer membrane fragments are separated from the guanidine hydrochloride supernatant by centrifugation at 48,000 x g for 30 min. The guanidine hydrochloride soluble fraction is dialyzed exhaustively at 4°C against several changes of distilled water over a period of 4 days. The nondialyzable fraction is centrifuged at 48,000 x g for 30 min. This water soluble fraction contains HP-protein and shows no morphologically recognizable units or fragments.

5.2. Comparison of HP-protein from outer wall vesicles and from single layer arrays

Whether or not the large, light arrays are truly single protein arrays can be tested by isolating those arrays by sucrose density gradient centrifugation. Pure protein arrays have density about 1.33 and would band at the bottom of a 60% sucrose solution. Gel electrophoresis can be applied to test the purity of the array and determine the molecular weight [Glaeser et al., 1979].
Circular dichroism analysis of purified HP-protein, isolated as described above, showed that the alpha-helix content of the protein molecule is about 20%, beta-sheet about 20%, and random coil about 60% [Wu, Wennie, unpublished results]. Circular dichroism analysis may also be applied to HP-protein isolated from single layer arrays to see if there are any changes in terms of the secondary structure.

Structural analysis with electron microscopy can be carried out with outer wall vesicles, which show moire patterns as described in chapter 2 (see Fig. 2-11b,c,d). We can then compare the results with those from the single layer arrays and see if there are structural changes, at least for the low resolution range.

5.3. Comparison of the results between the frozen hydrated and NSFT method

First of all, the reason why the NSFT method can give high resolution is discussed here. I speculate that air drying with negative stain still renders the specimen hydrated, or partly hydrated, and preserves high resolution structures. Negatively stained catalase can be preserved to 8 Å resolution, as seen by electron diffraction [Glaeser, 1971]; and negatively stained stacked disk aggregates of tobacco mosaic virus protein can be preserved to 12 Å image resolution [Unwin, 1974]. These 2
results indicate on the one hand that air drying with stain still preserves some high resolution specimen structures; on the other hand, the limited resolutions that were obtained might be explained by the fact that the specimens were put in the high vacuum column at room temperature, and the specimens therefore became "vacuum dried" instead of "air dried". Furthermore, I suggest that radiation damage is reduced due to the low dose (about 8 electrons/Å²), low temperature conditions, just as in the case of the frozen hydrated method. None of the radiation-induced "fixation" [Unwin, 1974] or "stain redistribution" [Unwin, 1974; Glaeser, 1971] events are going on to a great extent.

From Table 3-2 and Table 4-3, one can see that for a resolution below about 23 Å, the structure factors for the two cases, the NSFT and frozen hydrated method, are 180° out of phase, wherever a comparison can be made; between 23 and 13.70 Å, the structure factors can be either in phase or out of phase. A possible explanation is given below:

From the contrast transfer function theory, it is derived that

\[ F[I_{image}(x,y)] = (2/\pi \sigma B) F[V'(x,y)] \sin \gamma(\vec{s}), \text{ for } \vec{s} \neq 0. \]

where \( F \) indicates the Fourier transform, and
\[ V'(x,y) = \int V(x,y,z) \, dz \]

is the projected potential of the object. (see chapter 1 for details). Two assumptions are made:

1. \[ V_{F-H} = V_P + V_I \]
2. \[ V_{NSFT} = V_P + V_S \]

where \( V_{F-H} \) is the potential of the frozen hydrated specimen; \( V_{NSFT} \) is the potential of the specimen prepared by the NSFT method; \( V_P \) is the potential of the protein; \( V_I \) is the potential of ice; and \( V_S \) is the potential of the stain.

Given that the scattering power is in proportion to density as a good approximation, and that the density of protein, ice, and stain is \( \approx 1.3, 1, \) and \( k(>>1) \) respectively, it can be written that:

\[ V_P \propto 1.3 \, E_{nv}(V_P), \text{ at low resolution}; \]
\[ V_I \propto 1 - E_{nv}(V_P); \]
\[ V_S \propto k \, [1 - E_{nv}(V_P)]. \]

where \( E_{nv}(V_P) \) is the "envelope" of the protein.

It can then be derived that:

\[ F \{ V'_P \} \propto F \{ 1.3 \, E_{nv}(V_P)' \}, \text{ at low resolution}; \]
\[ F \{ V'_I \} \propto \frac{-F \{ E_{nv}(V_P)' \}}{s \neq 0}; \]
\[ F \{ V'_S \} \propto k \, F \{ E_{nv}(V_P)' \}, \text{ for } s \neq 0. \]

where \( V'_P \) is the projected potential of the protein;
$E_{nv}(V_p)'$ is the projection of the "envelope" of the protein; $V_s'$ is the projected potential of the stain.

From the linearity of integration and Fourier transform, it can be shown:

$$F[I_{F-H}] = \frac{2}{\lambda_c B} \left[ F[V_p'] + F[V_I'] \right] \sin \gamma$$

$$\propto 0.3 \left( \frac{2}{\lambda_c B} \right) [F[E_{nv}(V_p)']] \sin \gamma, \text{ (at low resolution, for } s \neq 0).$$

$$F[I_{NSFT}] = \frac{2}{\lambda_c B} \left[ F[V_s'] + F[V_p'] \right] \sin \gamma$$

$$\propto (1 - k) \times \frac{2}{\lambda_c B} [F[E_{nv}(V_p)']] \sin \gamma, \text{ for } s \neq 0.$$ 

where $I_{F-H}$ is the image intensity of the frozen hydrated specimen; and $I_{NSFT}$ is the image intensity of the specimen prepared by the NSFT method.

Assuming $\sin \gamma$ is the same and $k \gg 1$, it is seen that in the low resolution range, i.e., where $F[V_p'] = F[E_{nv}(V_p)']$ holds, the phases of the structure factors differ by $180^\circ$ in the two methods.

At higher resolutions, the above assumption, i.e., $F[V_p'] = F[E_{nv}(V_p)']$, may not hold, as the "internal" high resolution details of the protein structure "show up". The structure factors can thus be either in phase or out of phase for the two methods, in the higher resolution range.

However, this analysis is a mere speculation. More data is needed for a rigorous analysis, which is beyond the scope of this thesis. The one thing that will be very informative is to compare among results from frozen
hydration, NSFT, and negative stain method. By combining data from images taken at various defocus values, a comparison of results in the high resolution range can be made, which will be most interesting.

In the NSFT method it seems possible that both protein and stain contribute to the image contrast, and interpretation of images can be difficult. This may explain why the two computer processed images appear rather different (see Fig. 3-9 and Fig. 4-5).

A major advantage of the NSFT method is the fact that array recognition is made so easy by the stain, and as a consequence, high quality micrographs can be taken with ease. Therefore the NSFT method seems to be a very good way to assay for the existence of high resolution structures. To give a quantitative comparison, the micrograph shown in Fig. 4-1 is the result of just one night's work, whereas to obtain a good image of the frozen hydrated specimen, one year was spent. The difficulties are mainly due to the low contrast in the specimen prepared by the frozen hydrated method, and differentiation between single layer arrays and outer wall vesicles is very difficult. Now that it is seen that those large, single layer arrays do give high resolution images easily, as shown in chapter 4, the next thing to do is to isolate those single layer arrays in large quantity, as described in section 5.2, and do the structural analysis with frozen hydrated specimens.
of those single layer arrays.

5.4. Prospects for a high resolution map

The resolution of the micrograph shown in Fig. 4-1, an image of the specimen prepared by the NSFT method, seems to be limited by the sampling distance used, which was 2.5 \( R \) at the specimen. Likewise, the resolution of the frozen hydrated specimen, dealt with in chapter 3, seems to be limited by the sampling distance, 3.5 \( R \). A first thing that can be done is to use a sampling distance corresponding to 2 \( R \) at the specimen for the frozen hydrated method and the NSFT method, to see if higher resolution spots can be obtained.

As discussed in the end of chapter 3, both maps shown in Fig. 3-9 and 3-11 are incomplete representations of the full structure. A systematic combination of data from several image areas, taken at various defocus values, has to be applied through a detailed treatment of errors in electron imaging [Hayward and Stroud, 1981]. Even higher resolution maps should be obtained with the frozen hydrated specimen of large single arrays.

Eventually, a high resolution, 3-dimensional model can be obtained, by combining images taken at different tilt angles, of the frozen hydrated specimen of large single arrays. A 3-dimensional model for the outer wall vesicles can also be obtained. A great deal of crystallo-
graphic information can be derived as to the assembly of HP-protein to form arrays.

The content of this thesis is summarized below:

1. A method has been developed for obtaining large, well ordered crystalline arrays of the surface layer protein of *Aquaspirillum serpens*.

2. The diffraction pattern of the reconstituted arrays, regardless of their age or size, shows handedness (absence of mirror symmetry) in contrast to previous work, indicating that they are true single layer arrays, whereas the latter dealt with two sided vesicles.

3. A "NSFT" method was used for low dose imaging of the large arrays. 5.7 Å structural details were preserved in the images. Both stain and protein seem to contribute to the contrast.

4. 6.9 Å resolution was preserved and retrieved in the image of the frozen-hydrated specimen.
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